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Direct liquid chromatography determination of the reactive imine SJG-136 (NSC 694501)

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Abstract

SJG-136 (NSC 694501), 8,8′-[[(propane-1,3-diyl)dioxy]bis[(11aS)-7-methoxy-2-methylidene-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-*c*][1,4] benzodiazepin-5-one], which is being developed as a DNA-interactive antitumor agent, contains highly reactive imines in the diazepinone portions of the molecule. Water or alcohol adds readily to the imino moiety to form the corresponding carbinolamine or its alkyl ether, respectively. This sensitivity to protic substances poses a formidable challenge to the formulation and HPLC assay development for the compound. After studying the solution chemistry of SJG-136 and its potential interaction with various stationary phases, two reversed-phase liquid chromatographic assays for the compound have been developed. A direct assay that separates SJG-136 from its water or methanol adducts and an indirect assay that quantifies SJG-136 as its dihydrate adduct are reported. The latter method, which is more practical for drug development, has been validated. It is reproducible (R.S.D. < 2%), linear (r^2 = 0.9999) and accurate (within 98–102% recovery), with a lower detection limit of 2.5 ng.

Keywords: SJG-136; NSC 694501; Benzodiazepine; Water adducts; NMR; HPLC assay; Development; Validation

1. Introduction

SJG-136 (NSC 694501); 8,8′-[[(propane-1,3-diyl)dioxy]-bis[(11aS)-7-methoxy-2-methylidene-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-*c*] [1,4]benzodiazepin-5-one]) is a novel sequence-selective DNA-interactive minor-groove interstrand cross-linking agent that has shown significant activity in both in vitro and in vivo models [1–7]. It binds in the minor groove of DNA, predominantly at Purine-GATC-Pyrimidine sequences, cross-linking the two guanine residues on opposite strands via covalent aminal bonds between the N2-positions of the guanine residues and the C11/C11′-positions (CH=N) of SJG-136 molecules [1,2]. Interestingly, the adduct causes little distortion of the DNA helix which is thought to explain why SJG-136/DNA adducts are relatively resistant to repair [2]. The adducts are known to significantly raise the melting temperature of the DNA helix, block transcription in a sequence-dependant manner and inhibit the action of endonucleases having a Purine-GATC-Pyrimidine sequence at their recognition site [3,7]. SJG-136 is currently being studied in Phase I clinical investigations in both the USA (NCI) and the UK (Cancer Research UK), and for this reason a robust HPLC-based assay method was required.

The imine bonds in the diazepinone portions of SJG-136 (Fig. 1a) have significant reactivity towards protic (i.e. nucleophilic) substances via an addition reaction. Water or methanol adds easily across them, resulting in the formation of carbinolamines or carbinolamine methyl ethers. The addition of protic solvents is reversible under anhydrous conditions, however. This reversible reaction poses a challenge

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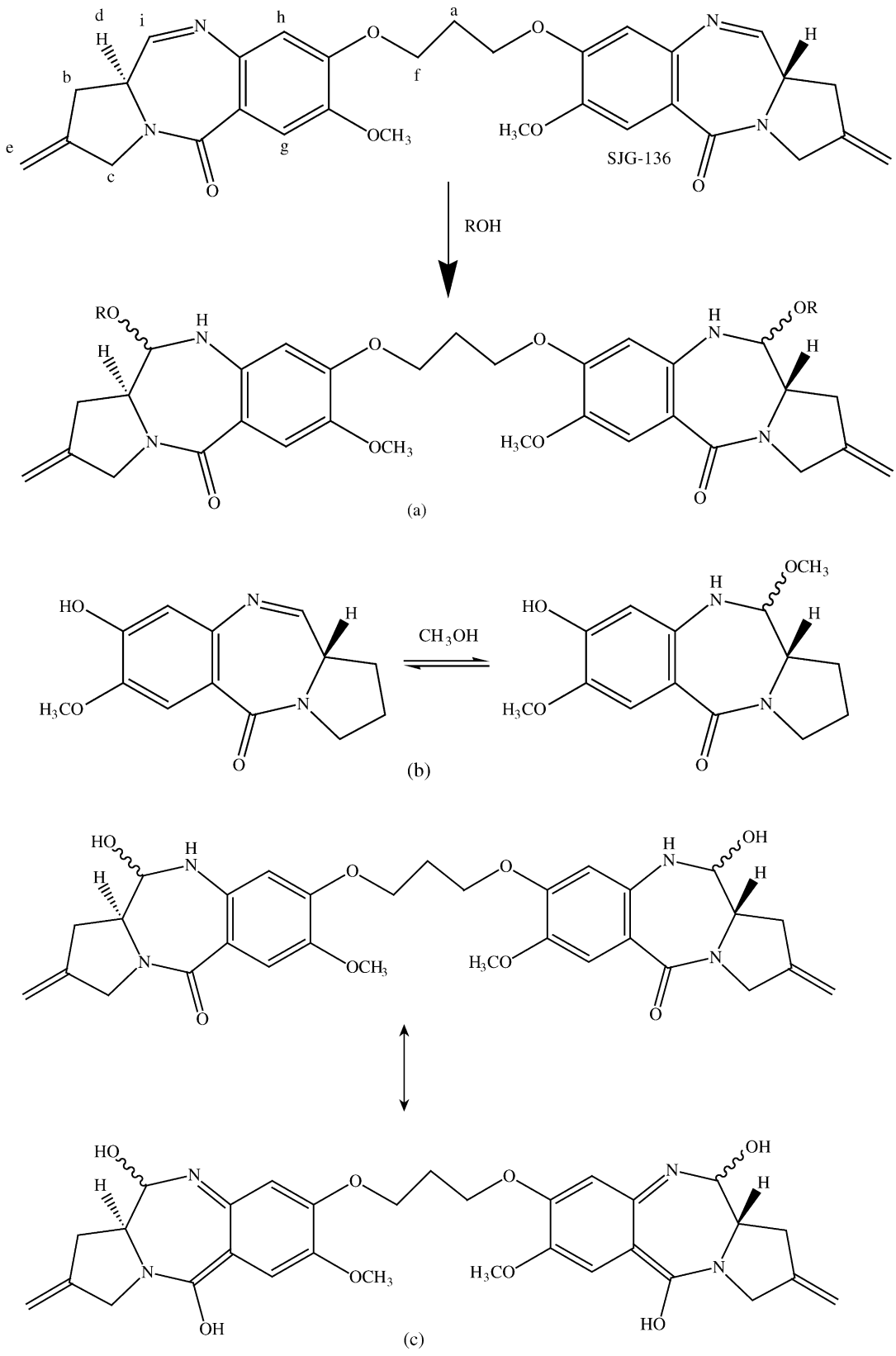

Fig. 1. Chemical structure of SJG-136: (a) formation of adducts (R = H or CH_3), (b) carbinolamine methyl ether of DC-81, and (c) possible keto-enol tautomerism of SJG-DHA.

to the formulation and HPLC analysis of SJG-136. Many reversed-phase HPLC assays for benzodiazepine agents have been reported [8–14], and a reversed-phase HPLC assay procedure for an analog of SJG-136 (DSB-120) has been described in the literature [3]. These assays use silica based HPLC columns and aqueous mobile phases, and no data is provided to indicate if the benzodiazepines are being assayed as the intact molecules or their hydrate adducts. We present here the initial development of a non-aqueous HPLC assay for intact SJG-136, a comprehensive study of the solution chemistry of the analyte, and the eventual development of a reversed-phase HPLC assay for the dihydrate adduct of SJG-136.

2. Experimental

2.1. Chemicals and reagents

SJG-136 (NSC 694501), lots Z/4, Z/5, and Z/6 were provided by the National Cancer Institute (Bethesda, MD, USA). Monobasic potassium phosphate (KH_2PO_4) and HPLC grade acetonitrile (ACN) were purchased from Mallinckrodt (Paris, KY, USA). Triethylamine, morpholine hydrochloride, and piperidine (99%) were obtained from the Aldrich Chemical (Milwaukee, WI, USA). Water was purified through a Millipore Super-Q Pure Water System (Waltham, MA, USA). The internal standard (IS), *p*-nitrotoluene from Sigma Chemical (St. Louis, MO, USA), was dissolved in ACN (0.1 mg/ml). Deuterated solvents, CD_3OD, CD_3CN, $CDCl_3$ and D_2O (all $\geq$ 99.8% atom D), for NMR experiments were purchased from Aldrich (Milwaukee, WI, USA).

2.2. HPLC

An Agilent 1100 HPLC system (Wilmington, DE, USA) was used. For assay, detection wavelength was set at 320 nm unless otherwise noted. For monitoring the UV profiles of forced decomposition products, and collection and processing of data, an Agilent ChemStation was used.

2.2.1. For intact SJG-136

SJG-136 sample solutions were prepared in ACN with brief sonication (1 mg/ml). The sample solutions (10 μl) were loaded onto two Phenomenex (Torrance, CA, USA) Ultracarb ODS, 5 μm, 250 mm × 4.6 mm i.d. stainless steel columns, joined in tandem. Chromatographic separation was carried out at 23 ± 1 °C by isocratic elution at 1 ml/min for 20 min with dry ACN containing 1% piperidine (v/v).

2.2.2. For the dihydrate adduct (SJG-DHA)

SJG-DHA sample solutions were prepared by first dissolving SJG-136 with 1 min sonication in ACN or the IS solution (0.1 mg/ml) followed by 1:1 dilution with water and sonication for 2–3 min to form the test solutions (0.2 mg/ml). Test solutions (10 μl) were loaded onto a Supelco (Bellafonte, PA, USA) Discovery RP Amide C16, 5 μm, 150 mm × 4.6 mm i.d. stainless steel column. Chromatographic separation was carried out at 23 ± 1 °C by isocratic elution at 1 ml/min with a 23:77 mixture of ACN and 20 mM KH_2PO_4 (pH unadjusted at 4.6) for 30 min, unless otherwise noted.

2.2.3. HPLC–MS

HPLC–MS was carried out on a ThermoQuest system consisting of a Surveyor LC pump, a Surveyor autosampler and a Finnigan LCQ-DUO mass spectrometer (San Jose, CA, USA). The mass spectrometer was equipped with an electrospray ionizations (ESI) probe operating at atmosphere pressure. The LC conditions used for identification of SJG-136 mono- and di-hydrates was the same as described in Section 2.2.1 (above). For the identification of impurities and decomposition products in the aqueous HPLC system, a Discovery RP Amide C16 column (5 μm, 150 mm × 4.6 mm i.d) was used with a mobile phase of ACN/water at 0.5 ml/min. The mobile phase was programmed to run at 22% ACN from 0–40 min, linearly ramped-up to 60% from 40–50 min, then re-equilibrated at the initial condition (22% ACN) for 10 min before the next run.

2.3. NMR experiments

The NMR experiments were carried out using a Varian Mercury Vx-Works Powered 300 (300 MHz) instrument. The SJG-136 samples were prepared in CD_3CN (Fig. 2a), CD_3CN plus CD_3OD (Fig. 2b), CD_3OD (Figs. 2c and 3a) and CD_3CN plus D_2O (Figs. 2d and 3b) at 1–5 mg/ml with brief sonication.

3. Results and discussion

3.1. NMR study on dihydrate adduct formation and solution chemistry of SJG-136

Fig. 2 presents the proton NMR of SJG-136 (a) in CD_3CN, (b) in CD_3CN plus 6% CD_3OD, and (c) in CD_3OD. With the exception of small impurity signals (marked*), the NMR of the CD_3CN solution (Fig. 2a) is consistent with the imine form of SJG-136. Spectral assignment is depicted in the figure, and the structure and labels are shown in Fig. 1a. The imine proton (doublet at 7.6 ppm) in each half of the dimer is clearly observed along with the two aryl protons at 6.8 and 7.4 ppm. NMR spectra obtained from this solution over a period of 24 h at room temperature remained unchanged. The NMR spectra of the CD_3CN solution, after the addition of two drops (6%) of CD_3OD (Fig. 2b), and that of the CD_3OD solution (Fig. 2c) are essentially identical. Both show the disappearance of the imine doublet (7.6 ppm) and the aryl signals (6.8 and 7.4 ppm). In their place are enhancements of the small impurity signals at around 7.3, 7.1, 6.4, 6.2, 5.1, 4.9, and 4.6 ppm. In fact, two sets of NMR signals are evident in these spectra. Each set is consistent with the *R* and *S* isomers

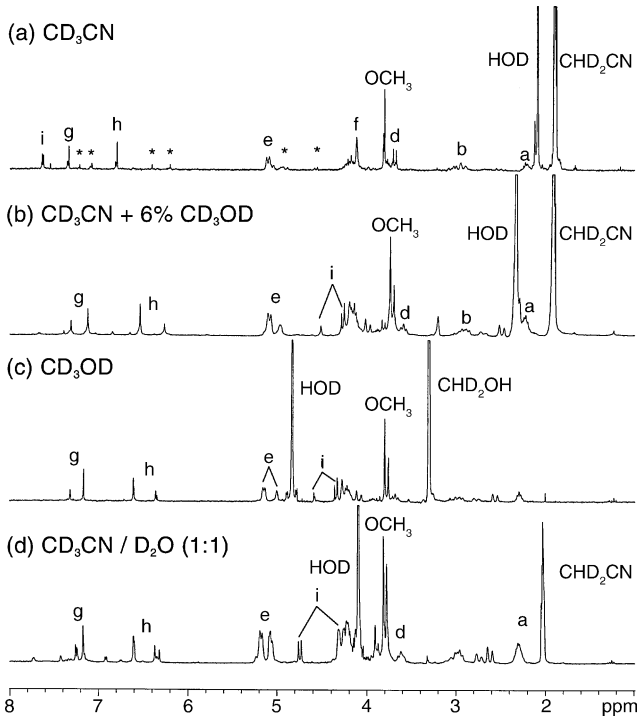

Fig. 2. Proton NMR spectra of SJG-136 dissolved in (a) CD_3CN, (b) CD_3CN followed by addition of 6% volume of CD_3OD, (c) CD_3OD, and (d) CD_3CN followed by addition of an equal volume of D_2O. See Fig. 1 for proton notations.

of a carbinolamine methyl ether resulting from addition of CD_3OD to the imine bonds of SJG-136 as depicted in Fig. 1a. Two-dimensional NMR (Fig. 3a) shows the carbinolamine CH protons at 4.55 (singlet) and 4.38 (doublet, $J = 9.3$ Hz) ppm. This is consistent with the literature [15] for the (*R*)- and (*S*)-carbinolamine methyl ether forms of DC-81 (Fig. 1b). Based on the literature, the set with the carbinolamine CH singlet at 4.55 ppm is the *R* isomer and the doublet at 4.38 ppm is the *S* isomer of the carbinolamine methyl ether.

The above experiment clearly demonstrates that SJG-136 can be converted to its adducts (carbinolamine methyl ether) after reaction with a protic substance such as methanol. Furthermore, less than 10% (v/v) of a protic solvent (ROH) in non-protic solvents (CD_3CN or $CDCl_3$) is sufficient to completely convert the imine to the carbinolamine alkyl ethers. Spectra from these solutions remained unchanged over 24 h. However, when the CD_3CN solution containing 6% CD_3OD or the CD_3OD solution alone was evaporated to dryness, the NMR spectra of the residue re-dissolved in CD_3CN or $CDCl_3$ were essentially identical to Fig. 2a, indicating that the adducts are easily converted back to SJG-136.

NMR spectra of solutions of SJG-136 in CD_3CN–D_2O mixtures (i.e. 6–50% D_2O) are identical to each other. A representative one is shown as Fig. 2d. It is similar to, but more complex than, the CD_3OD solution spectrum (Fig. 2c). The signals for the imino CH (7.6 ppm) are greatly reduced in favor of the new carbinolamine protons (4.2–4.4 and 4.6 ppm). Two-dimensional NMR (Fig. 3b), however, indicates a doublet at 4.6 ($J = 9$ Hz) for the (*S*)-isomer and a

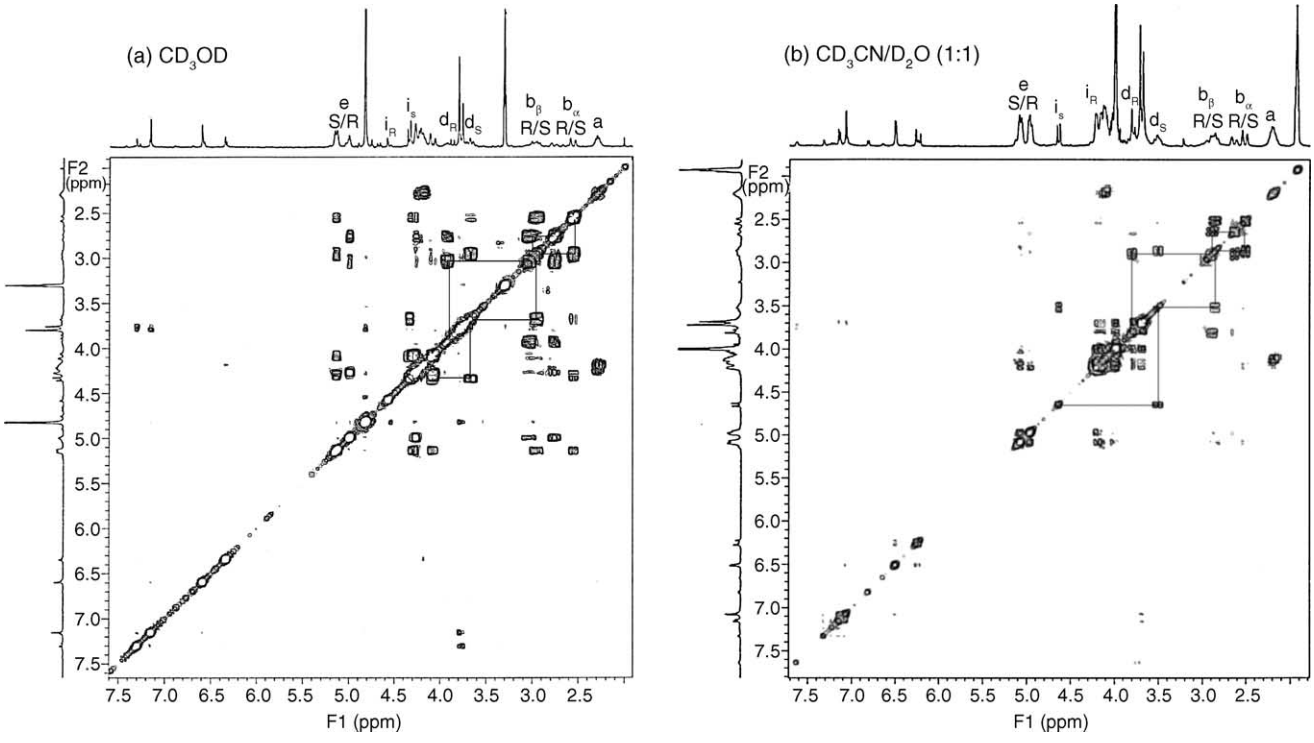

Fig. 3. 2-D NMR of SJG-136 in (a) CD_3OD and (b) CD_3CN followed by an equal volume of D_2O.

signal at 4.2 ppm ($J < 2$ Hz) for the (*R*)-isomer. The aryl protons at 7.4 and 6.8 ppm are replaced by signals at 7.0–7.2 and 6.2–6.6 ppm, respectively. Careful examination of the relative intensities and complexity of these signals reveals that the (*R*)-isomer exists as two species of similar intensity. It is possible that the aqueous adduct is further tautomerized (enol-keto forms) via the vinylogous amide (–OC–C=C–NH–) as depicted in Fig. 1c. NMR of a fresh solution of SJG-136 in CD_3CN/20 mM KH_2PO_4 in D_2O, which simulates the LC mobile phase in Section 3.3, is identical to those of other CD_3CN–D_2O solutions. NMR spectra of these solutions, obtained over a minimum of 24 h, remain unchanged compared to their initial spectra and suggest that the dihydrate adduct, once formed, is stable in solution for at least 24 h. When the 6% D_2O diluted CD_3CN solution was evaporated to dryness and the residue re-dissolved in CD_3CN, the NMR obtained was identical to Fig. 2a.

The foregoing experiments show that SJG-136 exists as the imino compound only in anhydrous non-protic solvents. In the presence of water or alcohol it exists predominantly as carbinolamine or carbinolamine alkyl ether adducts, respectively. However, the adducts are converted back to the imine under anhydrous or evaporative conditions.

3.2. HPLC assay for intact SJG-136

The reactivity of SJG-136 to nucleophiles poses a challenge to its analysis by HPLC. To minimize the risk of

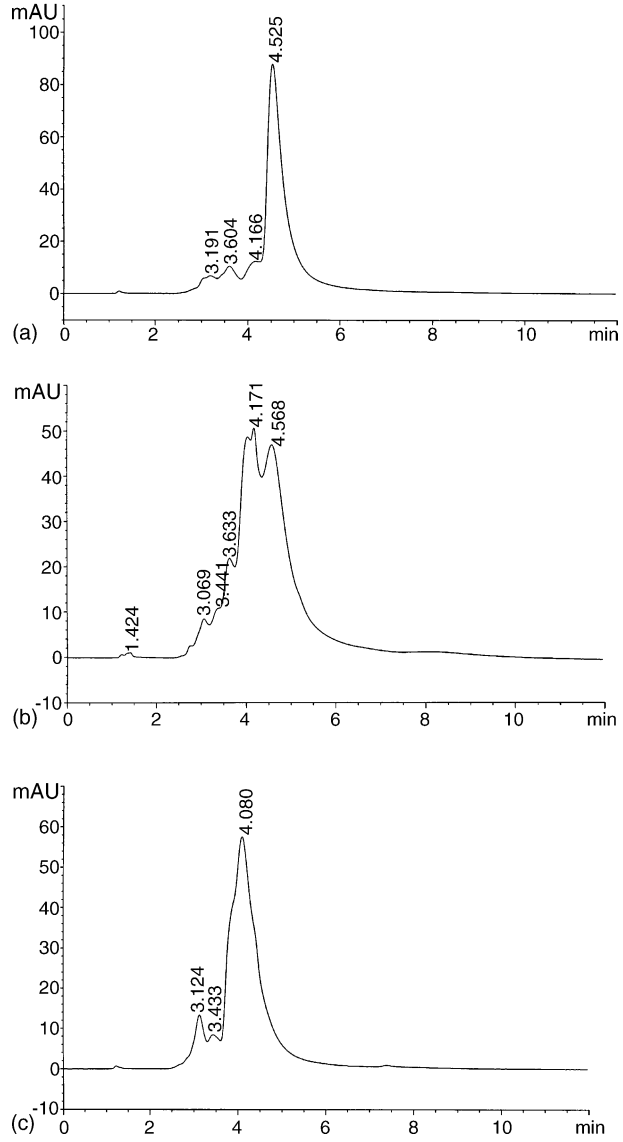

Fig. 4. Non-aqueous (ACN) mobile phase with 1% piperidine LC profiles of freshly prepared solutions of SJG-136 in (a) ACN; (b) 10% H_2O added to (a); and (c) 10% CH_3OH added to (a). Chromatography was carried out on a single column of Phenomenex Ultracarb ODS, 5 μm, 250 mm × 4.6 mm i.d..

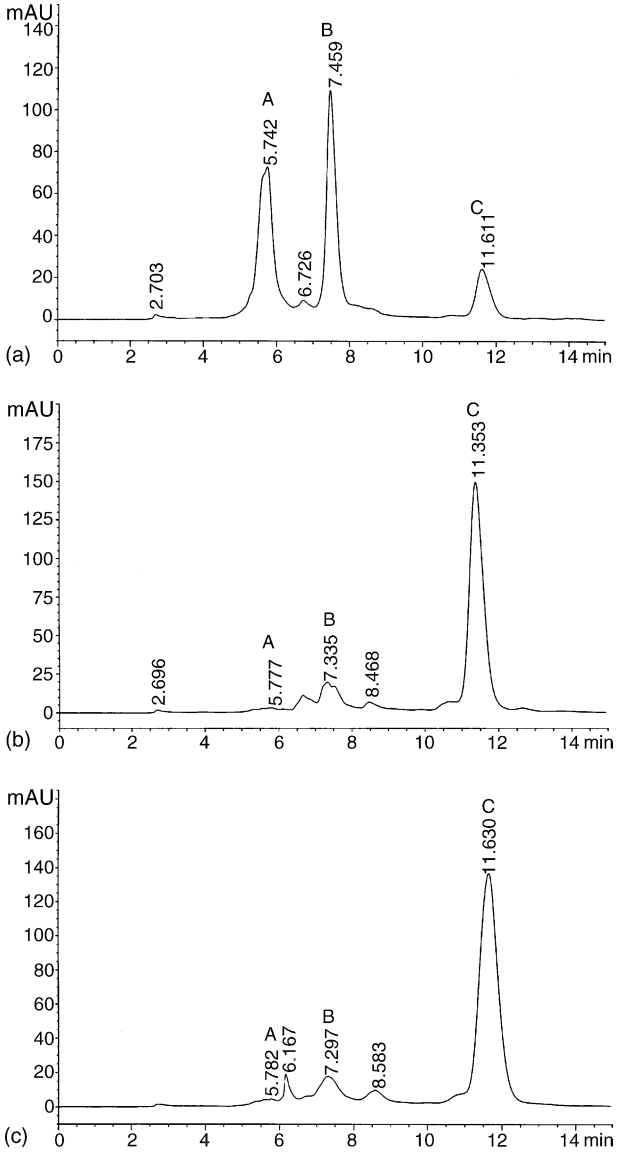

Fig. 5. Non-aqueous (ACN) mobile phase with 1% piperidine LC profiles of SJG-136 in (a) ACN, fresh; (b) ACN, standing for 4 h; (c) $CHCl_3$ fresh, the 6.2 min peak is due to $CHCl_3$. Chromatography was carried out on two columns of Phenomenex Ultracarb ODS, 5 μm, 250 mm × 4.6 mm joined in tandem.

SJG-136 reacting with residual silanol groups on silica based columns, two types of non-silica columns—the polymer based PRP column (Hamilton, Reno, NV, USA) and the carbon graphite Hypercarb column (Alltech, Dearfield, IL, USA) were first investigated. The PRP column, working under the reversed phase mechanism, is designed to retain analytes in the presence of high amounts of polar solvents. However, even the most polar aprotic mobile phase, ACN, gave no retention for SJG-136. The Hypercarb column gave either no retention with $CHCl_3$ or no elution with ACN as the mobile phase. When dioxane, a solvent of intermediate polarity, was used, on-column analyte decomposition was observed. As an alternative to non-silica based columns, a high carbon load (>25%) silica-based Ultracarb ODS column (Phenomenex, Torrance, CA, USA) was tried. With ACN as the mobile phase, SJG-136 eluted at $k' = 0.9$ (4.5 min, Fig. 4a) and was not well resolved from the apparent water or methanol adducts (Fig. 4b and c). Addition of organic bases such as triethylamine, morpholine or piperidine to the ACN increased column efficiency but decreased retention volumes for all peaks. To better retain the analytes, two Ultracarb columns were used in tandem. Of the three bases used, morpholine and piperidine gave better enhancement of efficiency. However, in a similar manner to dioxane, morpholine caused decomposition of SJG-136. As piperidine gave significant enhancement of the theoretical plate number (from <1000/column for neat ACN to 2500/column with 1% piperidine in ACN), and SJG-136 was stable in ACN containing piperidine, it was chosen as the mobile phase additive. This HPLC method, as described more fully in Section 2.2.1, is able to separate SJG-136 from its monohydrate, dihydrate and methanol adducts. The method development process is summarized in Table 1.

Table 1
Summary of method development

Column	Mobile phase	Result
PRP (Hamilton)	ACN	No retention
HyperCarb (Alltech)	$CHCl_3$	No retention
	ACN	No elution
	Dioxane	Partial decomposition of SJG-136
Ultracarb ODS (Phenomenex)	ACN	Broad peaks
	(Additives)	
	Triethylamine	Slightly improved peak shape
	Morpholine	Partial decomposition of SJG-136
	Piperidine	Significantly improved peak shape

Fig. 5a is the typical chromatogram of a freshly prepared ACN solution of SJG-136. When the solution was stored at room temperature, the earlier eluting peaks A and B at 5–8 min diminished in favor of the 12 min peak C (Fig. 5b),

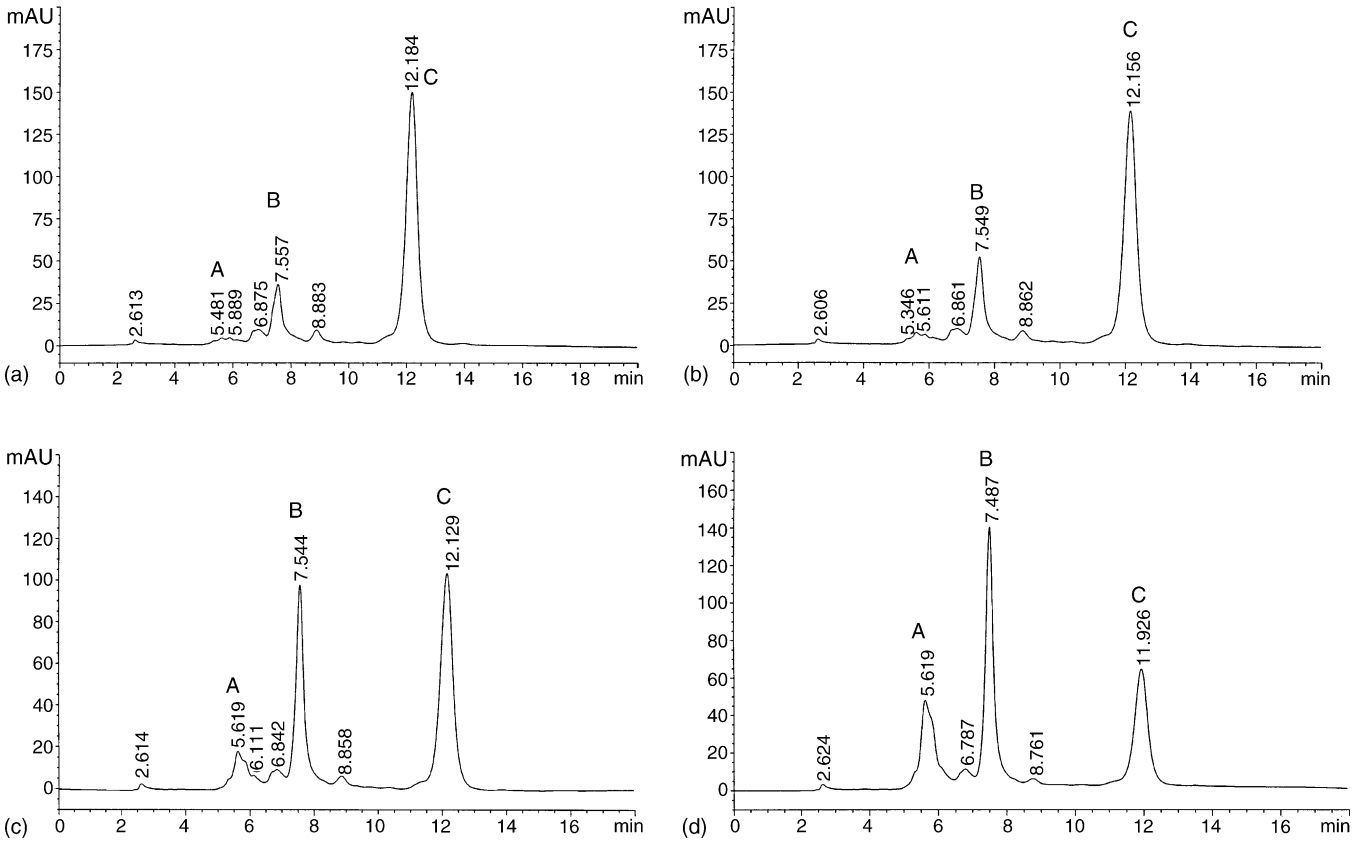

Fig. 6. Non-aqueous (ACN) mobile phase with 1% piperidine LC profiles of (a) SJG-DHA in ACN, standing for 4 days; (b) 0.03 ml H_2O added to 1 ml (a); (c) 0.06 ml H_2O added to 1 ml (a); (d) 0.1 ml H_2O added to 1 ml (a). Chromatography was carried out on two columns of Phenomenex Ultracarb ODS, 5 μm, 250 mm × 4.6 mm joined in tandem.

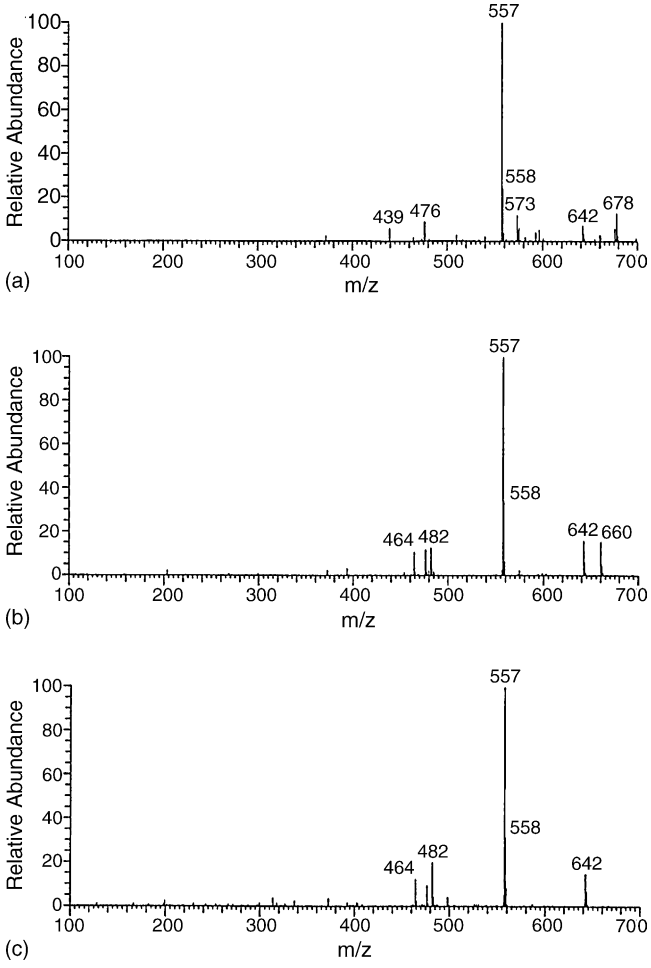

Fig. 7. Mass spectra of peaks A (a), B (b), and C (c) from Fig. 5a. Obtained with LC–MS. Chromatography was carried out on two columns of Phenomenex Ultracarb ODS, 5 μm, 250 mm × 4.6 mm joined in tandem, with mobile phase of dried ACN containing 1% piperidine (v/v) at 1 ml/min.

with the rate of conversion being concentration dependent. At 1 mg/ml, 4 h was required to reach a final constant composition, while 15 h were required for a 0.1 mg/ml solution. The final composition is analogous to that of a freshly prepared 1 or 0.1 mg/ml $CHCl_3$ solution (Fig. 5c), where the area of peak C is about 86%. It is noteworthy that peak C can be converted to peaks A and B with H_2O. When small increments of H_2O were added to a 4-days-old ACN solution, peak C diminished, first in favor of B and then A (Fig. 6a–d). This observation is consistent with assigning C as SJG-136 (imine form), and B and A as the mono- and dihydrate adducts, respectively. The UV spectra (from LC/UV) showed the λ_{max} of C (327 nm) was longer than that of B (323 nm) and A (319 nm), indicative of the more extended π-conjugation in C. The LC–MS data also confirmed that C, B and A are SJG-136 and its mono-and dihydrate adducts, respectively. Fig. 7 shows the mass spectra obtained with LC–MS. Apart from the dominant 557 ion for the pseudo-molecular ion ($M+H^+$) of SJG-136, MS of C (Fig. 7c) has a significant ion at 642 consistent with the $M+H^+$·piperidine complex. The MS of B (Fig. 7b) shows an additional 660 ion for the piperidine complex of the monohydrate adduct, and that of A (Fig. 7a) shows an additional 678 ion for the dihydrate adduct complex. The significant level of 557 ion in both cases is presumably due to conversion of the monohydrate or dihydrate forms back to the parent imine in the mass spectrometer probe. Similar experiments with CH_3OH indicate that the mono- and dimethanol adducts elute at 5.93 and 7.75 min, well resolved from the imine (11.97 min) but barely resolved from the corresponding water adducts at 5.6 and 7.5 min, respectively. Importantly, when peak C was collected and re-injected, the resulting chromatogram show only peak C, indicating that the earlier eluting components including A and B were not generated by the HPLC process. When the SJG-136 solution was freshly prepared with the mobile phase, relative peak area of A, B, and C were 63, 31, and 6%, respectively (Fig. 5a). Storing the solution (1 mg/ml) for 4 h at room temperature caused a slight change in favor of peak C (55, 35, and 10%, respectively for, A, B, and C), indicating that on-column conversion of the adduct to the imine was minimal (ca. < 4%). Thus, to monitor the actual sample composition of SJG-136, the test solution is best prepared freshly in the mobile phase.

Table 2
Relative (%) compositions of SJG-136 samples by "intact" HPLC assay

Lot	A (DHA)	B (MHA)	C (SJG)
Z/4	63.2	30.6	6.2
Z/5	83.1	15.3	1.6
Z/6	73.0	26.7	0.3

See Section 2.2.1 for HPLC conditions. A, B and C are peak designations as appearing in Fig. 5. DHA = dihydrate adduct, MHA = monohydrate adduct, SJG = intact SJG-136.

The non-aqueous HPLC assay described above has been utilized to study SJG-136 samples prepared by a purification procedure using reversed-phase HPLC with water/acetonitrile eluents. The results (Table 2) showed that they contained predominantly a mixture of hydrates.

The assay has also been used as a test for the crystallinity of the bulk drug substance, as the intact SJG-136 (diimino-form) is amorphous and has different solubility characteristics compared to the crystalline hydrate adducts. However, the sensitivity of SJG-136 to protic solvents would make its application to any type of bioassay impractical. Therefore, further development and validation of the assay was not pursued. Instead, an alternate HPLC assay was sought to determine SJG-136 as its dihydrate adduct (SJG-DHA).

3.3. HPLC assay of SJG-136 as the dihydrate adduct

The rationale for the assay of SJG-136 as SJG-DHA is the demonstrated ease of dihydrate formation in the presence of water. In addition, SJG-136 is expected to be present predominantly as the dihydrate in biological systems since it is formed so readily in aqueous environments. It was considered that a reversed-phase HPLC method with aqueous

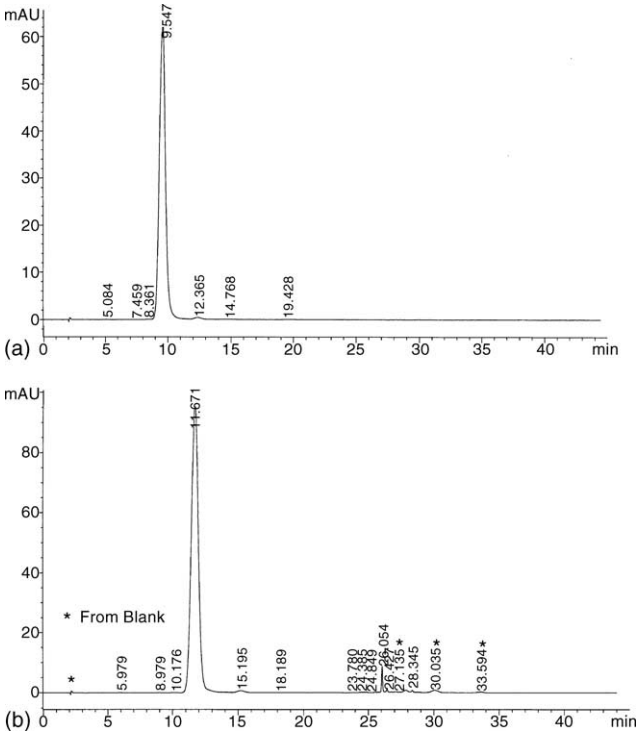

Fig. 8. Aqueous mobile phase LC profiles of a fresh solution of SJG-DHA in 1:1 CD_3CN–D_2O: (a) isocratic elution; and (b) isocratic elution for 23 min followed by linear gradient over 17 min to 55% ACN-buffer. Retention time of SJG-136 was delayed to 12 min due to slight variation in LC condition. The isocratic LC conditions: A Supelco (Bellafonte, PA) Discovery RP Amide C16 (5 μ, 150 × 4.6 mm i.d.) column with mobile phase of a mixture of ACN and 20 mM KH_2PO_4 (23:77).

mobile phase would ensure that the analyte stays as SJG-DHA during chromatography.

After experimentation with several ODS columns, a reversed-phase HPLC assay as described in Section 2.2.2., using a Supelco Discovery RP Amide C16 column, was used for the assay of SJG-136. Although the ODS columns gave reasonable chromatographic retention for SJG-DHA, the RP Amide C16 column gave the best separation of SJG-DHA from impurities and decomposition products.

Fig. 8 presents the chromatograms of a SJG-136 solution dissolved in CD_3CN–D_2O, where NMR data had confirmed that SJG-136 had been converted to SJG-DHA. The chromatogram (Fig. 8a) shows a major peak (9.6 min, 97.6% peak area) and a minor one (12.5 min, 0.6% peak area) along with several trace peaks (5.0, 7.5, 8.4, 14.8, and 19.4, totaling 0.2% peak area). In addition, several very late eluting trace impurity peaks totaling 1.6% of peak area were detected only after

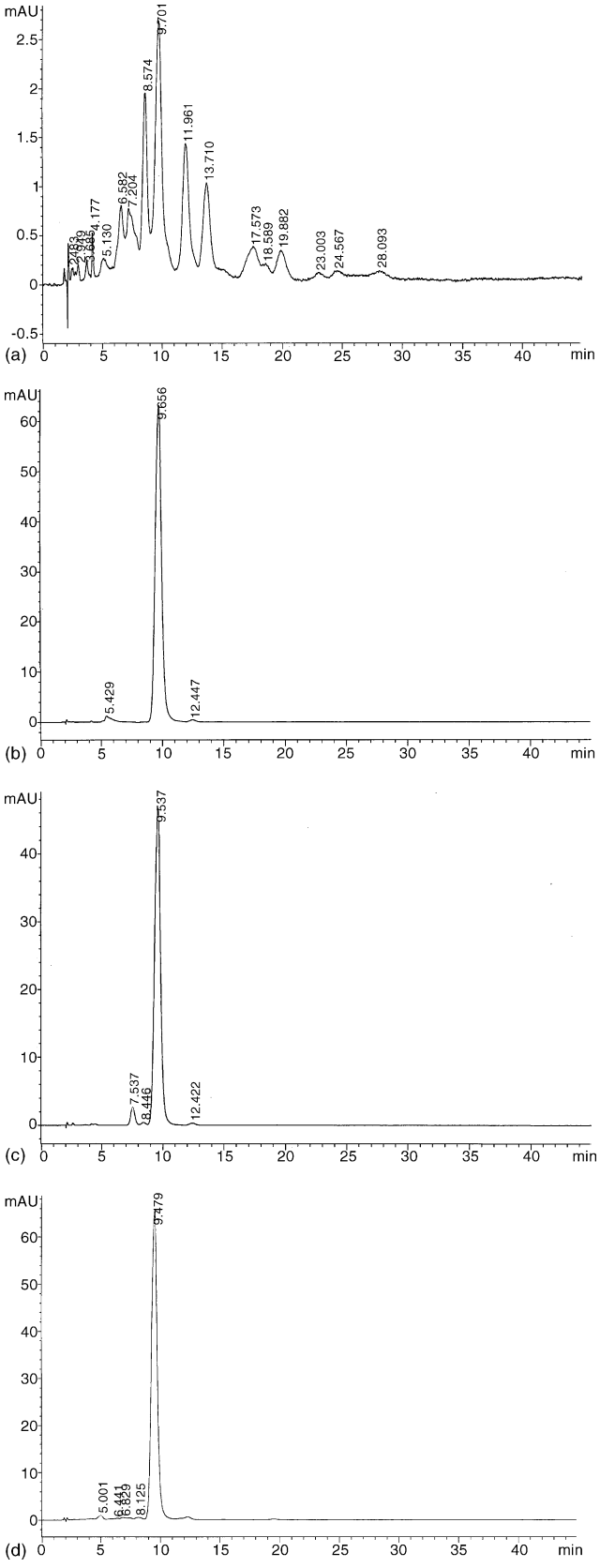

Fig. 9. Aqueous mobile phase LC profiles of solutions of SJG-DHA in (a) ACN–0.1N HCl (1:1), 24 h at room temperature; (b) ACN–0.1N NH_4OH (1:1) 24 h at room temperature; (c) ACN–H_2O (1:1), 4 h at 85 °C; and (d) SJG-136 bulk chemical, heated at 85 °C for 4 h then dissolved in ACN–H_2O (1:1). LC conditions: A Supelco (Bellafonte, PA) Discovery RP Amide C16 (5 μm, 150 mm × 4.6 mm i.d.) column with a mobile phase of a mixture of ACN and 20 mM KH_2PO_4 (23:77).

the mobile phase was changed by gradient elution to 60% ACN (Fig. 8b). This LC profile is identical to those obtained with fresh solutions of SJG-136 in 1:1 ACN/ H_2O or ACN/ 20 mM phosphate buffer (pH 4.6). The late eluting impurities, however, would not be eluted under the isocratic conditions alone and thus would not interfere with subsequent isocratic runs. Thus, for the assay of SJG-DHA, 25–30 min of isocratic elution is sufficient. For qualitatively monitoring the impurity profile, however, a linear gradient of the mobile phase to 60% ACN in 5 min and a plateau for 5 min was added to the end of the isocratic chromatography.

3.4. Stability of SJG-136 dihydrate adduct

Chromatograms of SJG-136 in the mobile phase of the SJG-DHA assay are identical to those of other ACN-aqueous solutions. Chromatograms from these aqueous solutions which were allowed to stand at $23 \pm 1\,^{\circ}C$ under normal laboratory conditions for at least 24 h were unchanged from the initial ones. Repeated injections of a 0. 18mg/ml solution resulted in peak area ratios (SJG-DHA/internal standard) of 2.24 (R.S.D. = 0.02%, $n = 5$) on the initial day, and 2.28 (R.S.D. = 0.01%, $n = 5$) after 24 h. This confirms that SJG-DHA is stable in ACN-aqueous solutions for at least 24 h. A 30 min stop-flow after injection did not alter the relative intensities of the major and minor peaks. In addition, when the major peak was collected and immediately re-injected, none of the minor peaks were observed in the resulting chromatograms. These results indicate that SJG-DHA is stable in neutral or mildly acidic (pH 4.6) aqueous solutions, and that the minor and late eluting peaks are not generated during the chromatographic process.

3.5. Validation of the HPLC assay

Specificity of the HPLC assay was demonstrated by forced decomposition of the SJG-DHA solution. In strongly acidic

Table 3
Aqueous mobile phase LC peak data

Peak	Retention	M + H (LC–MS)	Proposed structure
1	2.1 min	na	Hydrolytic product
2	2.6	317[a]	A
3	4.7	607	B
4	5.1	591	C
5	5.4	na	Hydrolytic product
6	7.5	na	Hydrolytic product
7	8.4	589	D
8	9.5	557[a]	SJG-136
9	12.0	573[a]	E
10	12.4	482[a]	F
11	13.7	na	Acid decomposition
12	14.7	na	Unknown impurity
13	17.6	na	Acid decomposition
14	19.4	na	Acid decomposition
15	22.2		*p*-Nitrotoluene, IS

Retention data are from Figs. 8 and 9.

[a] These imines existed as water adducts (carbinolamines) in sample solution and during chromatography. They reverted back to the imines during MS.

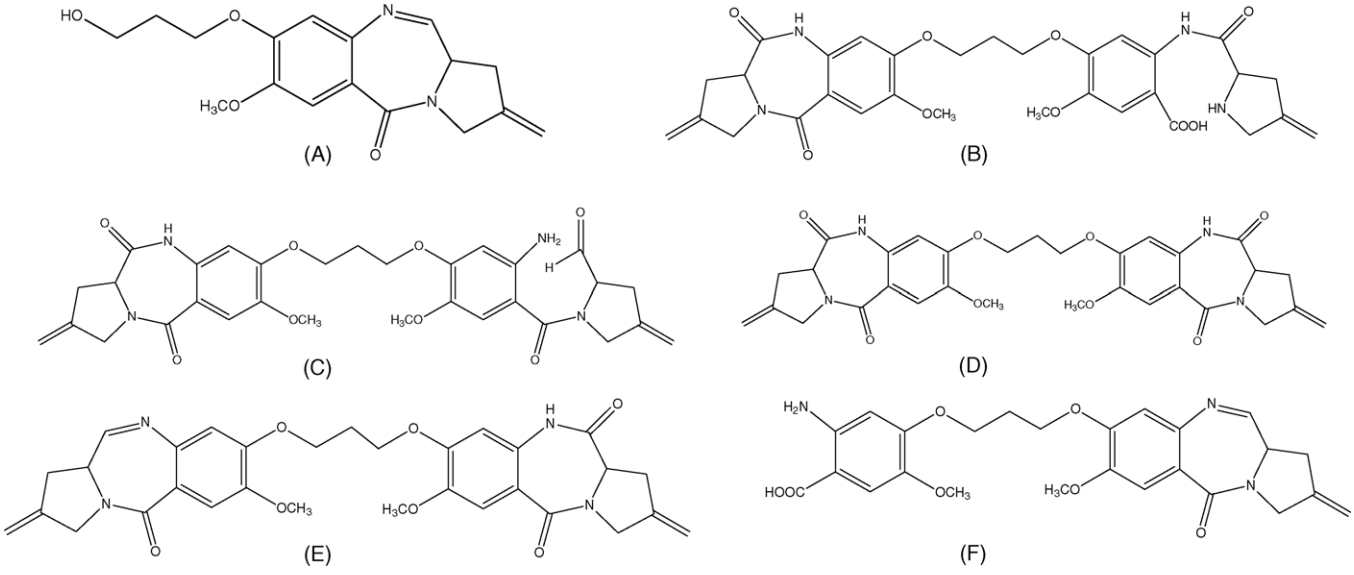

Table 4
System suitability

Chemical	SJG-DHA	*p*-Nitrotoluene (IS)
Retention time (min)	9.5	22.2
Capacity factor, k'	3.7	10.1
Resolution (S/IS)	11.7	
Asymmetry at 10% peak height	1.11	1.04
Number of theoretical plates/column	2480	6022

solution (ACN/0.01N HCl, 1:1), SJG-136 showed a significant reduction of the major 9.6 min (SJG-DHA) peak after 24 h storage at room temperature (23 ± 1 °C). This reduction was accompanied by the generation of numerous peaks eluting before and after the SJG-DHA peak (Fig. 9a). In neutral and basic aqueous solutions, however, SJG-DHA was fairly stable. Only a tiny 5.4 min degradation product was formed when a basic solution (ACN/0.1N NH_4OH, 1:1) was allowed to stand at room temperature (23 ± 1 °C) for 24 h (Fig. 9b). A minor amount of a 7.5 min degradation product appeared when a neutral solution (ACN/H_2O, 1:1) was heated for 4 h at 85 °C (Fig. 9c). As a bulk chemical SJG-136 was reasonably stable as well. Fig. 9d shows the chromatogram obtained from a solid sample of SJG-136, which had been heated at 85 °C for 4 h. It was essentially identical to that of the untreated bulk (Fig. 8a). Decomposition products did not interfere with the SJG-DHA and IS peaks, indicative of the specificity of the method. Table 3 lists the chromatographic retention data, pseudo-molecular ions from LC–MS, and the structures proposed to correspond to the peaks observed for SJG-136 and its forced decomposition products.

The system suitability data is presented in Table 4. The HPLC assay has a minimum linearity range of 0.05–0.80 mg/2 ml in 1:1 ACN/H_2O. The linear curve obtained from 6 test solutions (0.05, 0.15, 0.25, 0.35, 0.50 and 0.80 mg/ml) was $y = 6.4825x - 0.0094$. The correlation coefficient r^2 was 0.9999. The accuracy is evident from the recovery % at each of the three concentration levels, with three replicates at each level (Table 5). Recovery is calculated by comparing the theoretical and nominal concentrations. The theoretical concentration was obtained from the calibration curve (above) using the measured *R* (ratio of SJG-DHA peak area/ internal standard peak area). The average recovery was 100.6%. The intra-day precision is shown for three concentration levels (five replicates at each level). The R.S.D. at all concentration levels is ≤ 0.8%. The inter-day precision was estimated by analyzing a sample solution on three separate days, with five replicates each day. The R.S.D. of the total number of runs is 1.94% (Table 6). Therefore, the assay is sensitive, with a lower detection limit of 2.5 ng and a lower quantitation limit of 8.3 ng, based on three and ten times the observed signal to noise ratio.

Table 5
Linearity and Accuracy

W (mg/2 ml)	*R*	Recovery (%)	R.S.D. (%) ($n = 3$)
0.1484	0.9516	101.94	0.02
0.3488	2.2504	99.74	0.76
0.799	5.1619	100.14	0.28

The linear curve obtained from test solution of 0.05–0.8 mg/2 ml was $y = 6.4825x - 0.0094$, correlation coefficient was $r^2 = 0.9999$.

Table 6
Precision

		W (mg/2 ml)	Average *R*	R.S.D. (%) ($n = 5$)
Intra-day		0.0455	0.2916	0.38
		0.3488	2.2365	0.80
		0.799	5.1673	0.68
Inter-day	Day 1	0.3488	2.2365	0.80
	Day 2	0.3488	2.2865	0.32
	Day 3	0.3488	2.3184	2.02
	Day 1–3		2.2805	1.94 ($n = 9$)

4. Conclusions

Therapeutic agents that possess chemically reactive groups such as imines pose special challenges with regard to HPLC assay development. Due to the reactivity of the imine groups of SJG-136 towards protic solvents, the choice of HPLC column and mobile phase is very limited. Two reversed-phase HPLC assays have been developed: one for the assay and separation of SJG-136 from its adducts, and the other for the assay of SJG-136 in its dihydrate adduct (SJG-DHA) form. Due to practical considerations, only the latter assay was validated. This assay is suitable for both bulk analysis of SJG-136 and for formulation development, although it will not distinguish between mono- and di-hydrate adducts.

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